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Macrophage-Derived Factors Stimulate Optic Nerve Regeneration

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After optic nerve injury in mature mammals, retinal ganglion cells (RGCs) are normally unable to regenerate their axons and undergo delayed apoptosis. However, if the lens is damaged at the time of nerve injury, many RGCs survive axotomy and regenerate their axons into the distal optic nerve. Lens injury induces macrophage activation, and we show here that factors secreted by macrophages stimulate RGCs to regenerate their axons. When macrophages were activated by intravitreal injections of Zymosan, a yeast cell wall preparation, the number of RGC axons regenerating into the distal optic nerve was even greater than after lens injury. These effects were further enhanced if Zymosan was injected 3 d after nerve crush. In a grafting paradigm, intravitreal Zymosan increased the number of RGCs that regenerated their axons through a 1.5 cm peripheral nerve graft twofold relative to uninjected controls and threefold if injections were delayed 3 d. In cell culture, media conditioned by activated macrophages stimulated adult rat RGCs to regenerate their axons; this effect was potentiated by a low molecular weight factor that is constitutively present in the vitreous humor. After gel-filtration chromatography, macrophage-derived proteins $\geq$30 kDa were found to be toxic to RGCs, whereas proteins $<$30 kDa reversed this toxicity and promoted axon regeneration. The protein(s) that stimulated axon growth is distinct from identified polypeptide trophic factors that were tested. Thus, macrophages produce proteins with both positive and negative effects on RGCs, and the effects of macrophages can be optimized by the timing of their activation.

Key words: retina; ganglion cell; GAP-43; monocyte; trophic factor; cell culture

Introduction

The optic nerve (ON) has long served as a model system for understanding regenerative success or failure in the CNS. Under normal circumstances, mature retinal ganglion cells (RGCs) fail to regrow their axons distal to the site of optic nerve injury. Moreover, if axotomy occurs within the orbit, >95% of RGCs undergo apoptosis within 2 weeks (Mey and Thanos, 1993; Berkelaar et al., 1994). Regenerative failure is not inevitable, however. Ramon y Cajal (DeFelipe and Jones, 1991) described Tello's observation that mature RGCs can regenerate axons through a peripheral nerve (PN) graft sutured to the cut end of the optic nerve and concluded that "the regenerative failure of the central paths is... an accidental condition, due to the neuroglial environment." Expanding on this observation, Aguayo and colleagues (Richardson et al., 1980; David and Aguayo, 1981; Aguayo et al., 1991) showed conclusively that mature RGCs and other CNS neurons retain an intrinsic capacity for axon growth in an altered environment, sparking renewed interest in the factors that support or inhibit nerve regeneration.

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Several polypeptide trophic factors, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), GDNF, and fibroblast growth factor 1 (FGF-1), augment RGC survival after axotomy, but their effects are transient, and none has been reported to promote axon regeneration distal to an injury site (Carmignoto et al., 1989; Mey and Thanos, 1993; Cohen et al., 1994; Mansour-Robaey et al., 1994; Rabacchi et al., 1994; Di Polo et al., 1998; Koeberle and Ball, 1998). Overexpressing the anti-apoptotic protein Bcl-2 leads to long-term RGC survival after axotomy *in vivo*, but no axon regeneration (Chierzi et al., 1999). On the other hand, implanting a fragment of peripheral nerve into the vitreous (Berry et al., 1996) or injuring the lens (Fischer et al., 2000; Leon et al., 2000) stimulates RGCs to extend lengthy axons through the injury site into the distal optic nerve. If the optic nerve is transected and the two ends are sutured together, RGCs stimulated by lens injury are reported to extend axons back to the superior colliculus (Fischer et al., 2001). More modest levels of optic nerve regeneration have been achieved *in vivo* using angiotensin II (Lucius et al., 1998), antibodies to the myelin protein, NI-250 (Papadopoulos et al., 2002), or *C. botulinus* C3 enzyme to inactivate Rho-A (Lehmann et al., 1999).

The present study explores the role of macrophage-derived factors in axon regeneration. Lens injury leads to massive macrophage infiltration into the eye, and activating macrophages in a manner that does not affect the lens causes RGCs to regenerate their axons into the optic nerve (Leon et al., 2000). We show here that macrophages secrete factors that are neuroprotective for RGCs and promote axon regeneration. The strongest effect of macrophage activation occurs if they are activated a few days after

optic nerve injury, which leads to considerably stronger axon growth than after lens injury. In the more permissive environment of a peripheral nerve graft, macrophage activation enables RGCs to regenerate severed axons rapidly over long distances.

Materials and Methods

Two different surgical models were used in this study. One, a nerve crush model, allowed us to study axon regeneration in the native environment of the ON. These studies were performed at Children's Hospital (Boston, MA) with the approval of the Institutional Animal Care and Use Committee. The other model involved transecting the ON and grafting a segment of PN to the cut end of the optic nerve. This part of the study was performed at the University of Western Australia under a protocol approved by that institution.

Optic nerve crush and intraocular injections. Surgical procedures were similar to those described previously (Berry et al., 1996; Leon et al., 2000). Adult male Fisher rats (Charles River Laboratories, Wilmington, MA), 200–250 gm, were anesthetized by intraperitoneal injection of ketamine (60–80 mg/kg) and xylazine (10–15 mg/kg). A 1–1.5 cm incision was made in the skin above the right orbit. The ON was exposed under an operating microscope, and its dura was opened longitudinally. Using angled jeweler's forceps (Dumont #5; Roboz, Rockville, MD), the ON was crushed 2 mm behind the nerve head for 5 sec, avoiding injury to the ophthalmic artery. Nerve injury was verified by the appearance of a clearing at the crush site; the vascular integrity of the retina was verified by fundoscopic examination after dilating the pupil with atropine. Intraocular injections were made at the posterior aspect of the eyeball using a 30 gauge needle, with care taken to avoid damaging the lens. Animals were injected intravitreally either with 5 μl of vehicle (PBS) on the same day as nerve injury ($n = 4$) or 3 d later ($n = 4$) or with Zymosan A, a yeast cell wall suspension that is a potent macrophage activator (12.5 μg/μl in PBS) (Sigma, St. Louis, MO) ($n = 20$). Zymosan was sterilized at 90°C for 10 min and injected either 7 or 3 d before nerve injury, or on the same day as nerve injury, or 3 or 7 d afterward ($n = 4$ per group). Rats in all groups survived 14 d after nerve crush. In three cases with intravitreal Zymosan injections, cholera toxin subunit B (CTB) (2.5 μg/μl in PBS, 5 μl; List Biologic, Campbell, CA) was injected into the vitreous 1 d before the animal was killed to verify that axons in the distal ON originated in viable RGCs.

Peripheral nerve grafts. Surgery was performed on adult Fisher rats (Animal Resources Center, Western Australia) under halothane anesthesia (induction 5%, maintenance 2% in a 1:2 O_2/N_2O mixture). The dura of the left ON was opened, and the nerve was completely transected intraorbitally 0.5 mm behind the optic nerve head. A 1.5 cm segment of autologous PN was obtained from the peroneal branch of the left sciatic nerve and transplanted onto the ON stump using 10/0 suture (Cui et al., 1999; Cui and Harvey, 2000). The analgesic buprenorphine was administered subcutaneously (0.02 mg/kg) at the time of surgery.

Animals with PN grafts were divided into five groups. Group 1 ($n = 6$) received intravitreal injections of saline immediately after ON–PN grafting and served as a control. Groups 2 and 3 ($n = 5$ per group) received intravitreal injections of Zymosan (12.5 μg/μl) either on the same day as the ON–PN graft or 3 d later. The fourth group ($n = 5$) received a lower dose of Zymosan (1.25 μg/μl) on the same day as the grafting procedure, and the fifth group ($n = 6$) received 1.25 μg/μl of Zymosan after a 3 d delay. Injections of Zymosan or saline were made through a pulled glass micropipette, avoiding damage to the lens. All animals survived for 3 weeks after ON transection and PN grafting.

Preparation for histology and immunohistochemistry. For the ON crush animals, the procedures for tissue preparation and immunostaining were similar to those used previously (Leon et al., 2000). Fourteen days after nerve crush, animals were given a lethal overdose of anesthesia and perfused through the heart with cold saline plus heparin followed by 4% paraformaldehyde. Eyes with the nerve segments up to the optic chiasm still attached were dissected free from connective tissue, postfixed overnight, and transferred to a 30% sucrose solution overnight with constant rocking (4°C). Frozen sections (15 μm) were cut longitudinally on a cryostat, thaw mounted onto coated glass slides (Superfrost plus, Fisher, Pittsburgh, PA), and stored at −20°C until further use.

Immunohistochemistry to visualize GAP-43-positive axons was performed as described (Leon et al., 2000) using an anti-GAP-43 antibody prepared in sheep [IgG fraction, 1:50,000 (Benowitz et al., 1988)] followed by a biotinylated secondary antibody, avidin–biotin complex (Vector Labs, Burlingame, CA), and diaminobenzidine enhanced with $NiCl_2$ (Vector Labs). In cases in which GAP-43 was visualized by immunofluorescence, the primary antibody was diluted 1:2500, and the secondary antibody was a fluorescein-conjugated anti-sheep IgG made in donkey (1:500, Alexa Fluor 488; Molecular Probes, Eugene, OR). Reactive macrophages were visualized with the ED-1 monoclonal antibody (1:200 dilution; Serotec, Raleigh, NC) in all cases. Secondary antibodies conjugated to distinct fluorophores were used to visualize ED-1-positive macrophages (Texas Red-conjugated anti-mouse IgG made in goat, 1:500; Molecular Probes) and GAP-43-positive RGCs (Alexa Fluor 488-conjugated anti-sheep IgG made in donkey, 1:500; Molecular Probes) in the same sections. Fluorescent sections were covered using Vectashield mounting medium (Vector Labs). For the cases with Zymosan injections and CTB anterograde tracing, an antibody to CTB (made in goat, 1:250 dilution; List Biologic) was used together with GAP-43 antibody, followed by the appropriate secondary antibodies conjugated to Texas Red (1:500; Vector Lab) and fluorescein, respectively.

Quantitation of axon growth in the optic nerve. Axon growth was quantified in four longitudinal sections through the ON for each case. Using a calibrated ocular to measure distance, we counted the number of GAP-43-positive axons crossing a line at distance d (0.5 or 1 mm) from the end of the crush site. By measuring the cross-sectional width of the nerve at the point at which the counts were taken, we converted axon counts into axon crossings per unit nerve width (axons per millimeter) and obtained the average of these over the four sections. Σa_d, the total number of axons extending distance d in a nerve having a radius of r, was estimated by summing over all sections of thickness t:

$$\Sigma a_d = \pi r^2 \times (\text{average axons/mm width})/t.$$

After calculating the total axon number in each case, we obtained group means and SEMs. ANOVA and Bonferroni's tests were performed to determine the significance of the group differences.

Quantitation of regenerating RGCs. For animals receiving PN grafts, 0.2 μl of 6% Fluorogold (Fluorochrome, Denver, CO) was slowly injected into the distal end of the graft 19 d after the original surgery. This retrogradely labeled RGCs that had regenerated their axons the full length of the graft. Two days later, animals were perfused with saline followed by 4% paraformaldehyde. Retinas were dissected out and postfixed with the same fixative for 1 hr, flat-mounted on slides after making relieving slits, coverslipped in Citifluor mounting medium (London, UK), and examined under a fluorescent microscope. The outline of each retina was drawn using a MD2 microscope digitizer (Accustage, Shoreview, MN), and a grid was placed onto the drawing. The number of FG-labeled RGCs per field (0.235 × 0.235 mm) was counted at each line-crossing point of the grid, and the average density of labeled RGCs in each retina was determined. Data were analyzed using ANOVA followed by Bonferroni's test or Dunnett's test for multiple comparisons. Student's t test was also used to compare two groups separately.

Immunostaining of whole-mount retinas. After counting FG-labeled RGCs, coverslips were carefully removed, and retinas were gently freed from the slides for immunostaining. One retina from the saline-treated group and two from the Zymosan-treated group were chosen for ED-1 immunostaining to visualize activated macrophages. Retinas from the intact right eyes of the same animals were used as normal controls. After three washes in PBS, retinas were blocked and permeabilized using 10% goat serum (Hunter Antisera, New South Wales, Australia) and 0.2% Triton X-100 for 1 hr and then incubated with ED-1 primary antibody (1:200; Serotec) for 2 d at 4°C. Retinas were incubated with a Texas Red-conjugated anti-mouse secondary antibody (1:100; Molecular Probes) at 4°C overnight and coverslipped in Citifluor mounting medium. For βIII-tubulin immunostaining, we used the monoclonal TUJ1 antibody (1:500; Babco, Richmond, CA), followed by a fluorescein-conjugated goat anti-mouse IgG (1:100; ICN Biochemicals, Costa Mesa, CA).

Macrophage-conditioned medium. Normal rat alveolar macrophages

(NR8383; American Type Cell Culture, Manassas, VA) were maintained in F-12K modified medium (Life Technologies, Gaithersburg, MD) with 15% fetal bovine serum and 1% penicillin–streptomycin for several days to weeks. Before being activated, macrophages were washed three times by being suspended in serum-free F-12K medium and centrifuged down to remove serum components. Resuspended macrophages were counted with a hemacytometer, and 10^7 cells were seeded in a 100 × 20 mm Polystyrene culture dish (Falcon, Bedford MA) in 10 ml of F-12K medium. Macrophages either were treated by adding Zymosan (1.25 mg/ml, final concentration) into the medium or were left untreated. After cells were incubated for 8 hr at 37°C in 5% CO_2, supernatants were collected, centrifuged (1500 × *g*) for 10 min to remove Zymosan particles and cell debris, and then put through a 0.2 μm low-protein binding filter (Pall-Gelman Laboratory, Ann Arbor, MI) to remove any remaining Zymosan particles. Protease inhibitors (Complete, Roche, Indianapolis, IN) were added to the supernatant (1 tablet/10 ml). Macrophage-conditioned medium (MCM) was concentrated using a 3 kDa molecular weight cutoff ultrafiltration membrane (Amicon/Millipore, Bedford, MA), and the fraction >3 kDa was aliquoted and stored at −80°C. For chromatographic analysis, MCM proteins >3 kDa were concentrated 83-fold by ultrafiltration.

Size separation of MCM. Gel filtration chromatography was performed on a Sephadex G-75 column. Sephadex G-75 beads (12 gm; Amersham Pharmacia Biotech, Piscataway, NJ) were swollen to 150 ml and packed in a column 1 × 90 cm. After the column was washed, 1 ml of concentrated MCM from Zymosan-activated macrophages was run through at 0.3 ml/min. The eluate was collected into 3 ml fractions that were stored at −20°C (with glycerol added to a final concentration of 30%) until further use. After glycerol was removed (by ultrafiltration), fractions were analyzed on 16% Tricine gels (Invitrogen) and bioassayed in RGC cultures as described below.

Retrograde labeling of RGCs from the superior colliculus. To distinguish RGCs in dissociated retinal cultures, cells were retrogradely labeled with FG. Adult Fisher rats, 200–250 gm, were anesthetized as above, a midline incision was made in the scalp, and a bone flap was opened above the occipital cortex. Posterior cortex was vacuum aspirated bilaterally to expose the superior colliculi and dorsal lateral geniculate nuclei. FG (2% in saline, 5 μl) was injected into the superior colliculi bilaterally, and small pieces of FG-soaked Gelfoam (Ethicon, Somerville, NJ) were inserted to cover the colliculi. The incision was closed, and animals were allowed to survive 1 week to permit the FG to be transported back to RGC somata.

Adult rat retinal cultures. Tissue culture plates (four wells; Nunc, Rochester, NY) were coated with poly-L-lysine (0.1 mg/ml, molecular weight >300,000; Sigma), rinsed with distilled water, air dried, and sterilized by exposure to UV light for 15 min. To prepare retinal cultures, FG-labeled animals were killed by an overdose of ketamine and xylazine administered intraperitoneally. Retinas were rapidly dissected from the eye cups and incubated at 37°C for 30 min in a CO_2 incubator in digestion solution containing papain (17 U/ml; Worthington, Lakewood, NJ) and L-cysteine (0.3 mg/ml; Sigma) in L-15 medium (Life Technologies) containing sodium bicarbonate (2.2 mg/ml). Retinas were then rinsed and triturated in L-15 containing bovine serum albumin (1 mg/ml; Sigma) and DNase (0.2 mg/ml; Sigma). Dissociated cells were passed through a strainer (40 μm nylon net; Falcon) and collected by centrifugation. Cells from two retinas were resuspended in 2 ml of L-15 containing $NaHCO_3$ (2.2 mg/ml), and 50 μl of this suspension was added to each well. The total volume in each well was brought up to 400 μl by adding 100 μl of a 4× concentrated serum-free defined medium (Medium E) described previously (Schwalb et al., 1995) and 250 μl of the concentrated experimental sample prepared in L-15 media with $NaHCO_3$. Samples were arranged in a pseudorandomized manner on the plates so that the investigator would not be aware of their identity when quantifying axon growth. Axon outgrowth and cell survival were assessed after maintaining plates in 5% CO_2 at 37°C for 3 d. FG-labeled RGCs were first identified under UV illumination and then viewed under phase-contrast to visualize axons. Axon growth was defined as the percentage of RGCs that extended axons more than or equal to two cell diameters in length. Cell survival was defined as the number of phase-bright RGCs per microscope field (400×) averaged over 30 fields per well. Values are given as the mean ± SEM of four replicate wells. Statistical significance was determined by ANOVA, Bonferroni's, and paired Student's *t tests*.

The trophic factors tested in these cultures included KC/GRO/CXCL1 (at 50 or 500 ng/ml; Chemicon, Temecula, CA); CNTF (rat recombinant, at 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, and 500 ng/ml; RDI, Flanders, NJ); epidermal growth factor (EGF) (human recombinant, at 1, 2, 1000 ng/ml; RDI); interleukin-6 (IL-6) (mouse recombinant, at 20, 50, 100 ng/ml; Alamone Labs, Jerusalem, Israel); FGF-2 (recombinant, 50 ng/ml; Alamone), pleiotrophin (human recombinant, 50 ng/ml; Alamone); cardiotrophin-1 (CT-1) (human recombinant, 100 ng/ml; Alamone), nerve growth factor (NGF) (mouse, 2.5S, 500 ng/ml; Alamone), BDNF (human, 5–50 ng/ml; Alamone), and forskolin (15 μM; Alamone).

Immunocytochemistry. Cells cultured on coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized, and blocked with 0.1% Triton X-100 and 5% goat serum for 30 min. Cells were incubated with a monoclonal anti-GAP-43 antibody (1:500 dilution, clone 9–1E12; Chemicon) at 4°C overnight, followed by a fluorescein-conjugated goat anti-mouse secondary antibody (1:500, Alexa Fluor 488; Molecular Probes) for 1 hr at room temperature. Coverslips were applied using Vectashield mounting medium. Controls were stained by omitting the primary antibody.

Results

Axon regeneration in the optic nerve is sensitive to the time of Zymosan administration

Lens injury, which increases the ability of RGCs to survive axotomy and regenerate their axons, is associated with macrophage infiltration into the vitreous (Leon et al., 2000). This led us to hypothesize that macrophages mediate the pro-regenerative effects of lens injury. In the first part of this study, we investigated whether macrophage activation fully mimics the effects of lens injury and whether there is an optimal time to activate macrophages to maximize the availability of axon-promoting factors when RGCs are most responsive. Macrophage activation was achieved by injecting Zymosan, a potent monocyte activator (Fitch et al., 1999), into the vitreous body without touching the lens. Injections were made on the same day as nerve injury, 7 or 3 d before nerve injury, or 3 or 7 d after nerve injury. Absence of lens damage was verified by an absence of cataract formation after 2 weeks (Fischer et al., 2000; Leon et al., 2000).

Controls given an intravitreal injection of PBS at the time of optic nerve crush showed no macrophage infiltration into the eye and no detectable GAP-43 in RGCs when examined after 2 weeks (Fig. 1*a*). These animals averaged <100 axons extending 0.5 mm past the crush site and fewer than half this number 1 mm distal to the injury site (Figs. 1*b*, 2). Additional controls injected with PBS 3 d after nerve injury showed similarly low amounts of growth (125.3 ± 21.5 axons at 500 μm; data not shown). In contrast, when Zymosan was injected into the eye, numerous ED-1-positive macrophages were seen in the vitreous and along the inner retinal surface in every case (Fig. 1*c*). RGCs showed an intense upregulation of GAP-43 in their somata and axons (Fig. 1*c*,*d*), and hundreds of GAP-43-positive axons extended well beyond the injury site, growing in tortuous trajectories down the length of the optic nerve (Figs. 1*d*, 2). GAP-43-positive fibers were double-labeled when the anterograde tracer, CTB, was injected into the eye (data not shown), confirming that the GAP-43-positive fibers distal to the injury site truly arise from RGCs (Leon et al., 2000).

We found previously that the number of intravitreal macrophages increases steadily during the first 7 d after stimulation and remains high for another 1–2 weeks (Leon et al., 2000). To make macrophage-derived factors available to RGCs before optic nerve crush, and hence potentially initiate axon regeneration before a

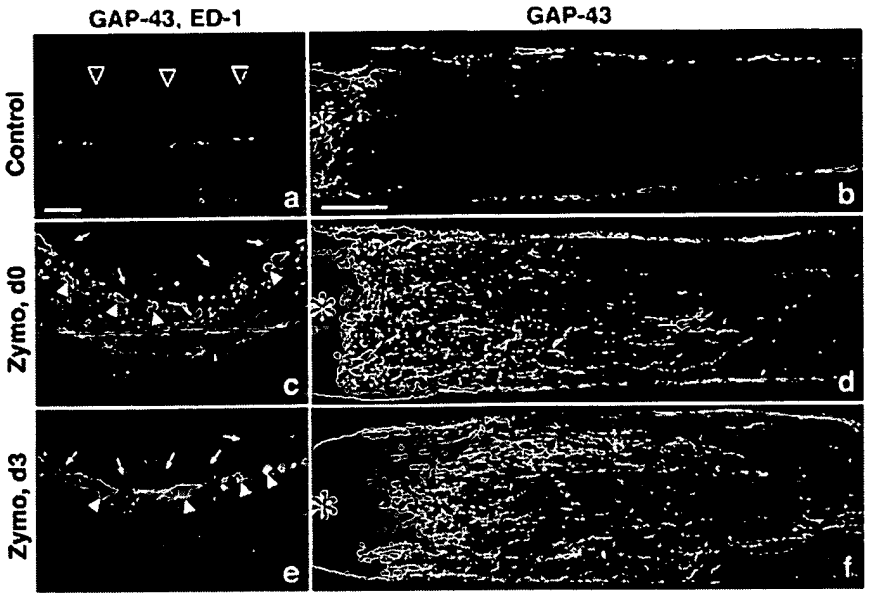

Figure 1. Macrophage activation induces axon regeneration in the rat optic nerve. Sections through the retina (*a*, *c*, *e*) or the optic nerve (*b*, *d*, *f*) were stained with antibodies to detect GAP-43 (*a–f*, *green* fluorescence) or the macrophage marker ED-1 (*a*, *c*, *e*, *red* fluorescence) 2 weeks after optic nerve surgery. *a*, GAP-43 is not detected in the GCL (*open arrowheads*) of animals that had received control PBS injections after optic nerve damage; ED-1 $^+$ macrophages are absent. *b*, Few GAP-43-positive axons extend past the injury site (*asterisk*) in the optic nerve. *c*, Zymosan injected into the vitreous the same day as nerve injury stimulates ED-1 $^+$ macrophages to infiltrate the eye and distribute near the GCL (*arrows*). GAP-43 expression is intense in RGC somata (*arrowheads*), and many GAP-43-positive axons extend into the distal optic nerve (*d*). *e*, Zymosan injections made 3 d after nerve injury result in high levels of GAP-43 in RGCs (*arrowheads*) and greater numbers of GAP-43-positive axons extending distal to the injury site (*f*). Scale bars: *a*, *c*, *e*, 250 μm; *b*, *d*, *f*, 200 μm.

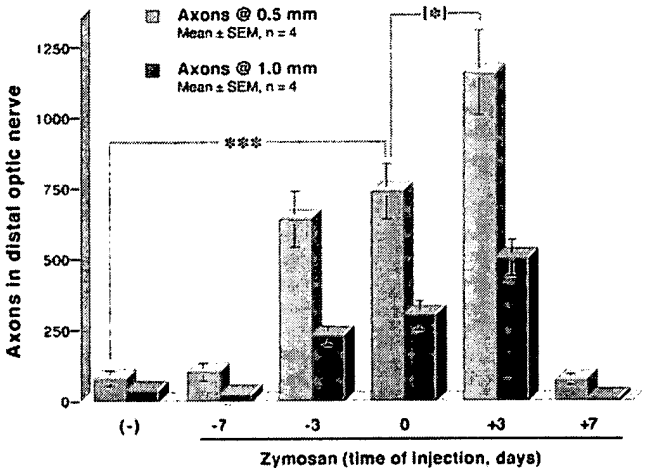

Figure 2. Sensitivity of axon regeneration to the time of macrophage activation. The *y*-axis shows the total number of axons measured at 0.5 and 1.0 mm distal to the crush site 2 weeks after nerve injury; the *x*-axis indicates treatments; (−) is the PBS injection control. Zymosan injected 3 d after optic nerve injury (+3) stimulated the highest levels of axon regeneration. [*] $p = 0.06$; *** $p < 0.001$.

scar forms, we injected Zymosan 3 or 7 d before nerve crush. Contrary to our expectations, introducing Zymosan 7 d before optic nerve surgery failed to stimulate axon regeneration, and Zymosan applied 3 d beforehand resulted in regeneration that was no higher than seen in animals that received injections on the same day as nerve crush (Fig. 2). This finding implies that something other than the overall number of macrophages determines whether RGCs are stimulated to regenerate their axons.

Delaying Zymosan injections for 3 d after nerve crush resulted in considerably greater levels of axon regeneration than were seen after immediate Zymosan injections (Figs. 1*f*, 2). Animals in which Zymosan injections were delayed by 3 d showed a 1.6-fold increase in axon growth relative to animals receiving immediate injections ($p = 0.06$) and a ninefold increase compared with controls with intravitreal injections of PBS delayed by 3 d ($p < 0.001$). On average, the longest axons in the delayed-Zymosan group grew 4.7 mm beyond the crush site in 2 weeks. This was twice as long as in animals that received immediate Zymosan injections (difference significant at $p < 0.05$; data not shown), despite the fact that the former group had 3 fewer days after macrophage activation to regenerate their axons at the time they were killed. A 7 d delay in Zymosan delivery resulted in no axon growth beyond the injury site (Fig. 2) (compare with control group; $p > 0.05$).

Axon regeneration in a peripheral nerve environment

Lens injury enhances the regeneration of RGC axons into a PN graft (Fischer et al., 2000). If macrophage activation is the principal mediator of lens injury-induced axon regeneration, we would expect that intravitreal Zymosan should increase axon growth in this paradigm. When Zymosan was injected on the same day as optic nerve transection and peripheral nerve grafting, >5700 RGCs extended their axons to the distal end of the graft; this is twice the number found in saline-injected controls (Fig. 3*a–c*) (difference significant at $p < 0.01$). Delaying Zymosan treatment by 3 d further increased the number of RGCs regenerating their axons the length of the graft by a factor of 1.5 relative to the group receiving immediate Zymosan injections (Fig. 3*a–d*) ($p < 0.05$) and by a factor of 3 over baseline ($p < 0.001$). Thus, either in the native optic nerve environment or in the more permissive environment of the PNS, the extent of axon regeneration is sensitive to the time of Zymosan injection. A 10-fold dilution in Zymosan concentration (1.25 μg/μl) resulted in somewhat fewer macrophages in the eye but induced as much regeneration as found with the original concentration used (Fig. 3*a*). RGCs that extended axons part way through the graft or picked up only low levels of FG would not be labeled in any of these groups.

The survival of RGCs was evaluated using the TUJ1 antibody, which recognizes neuron-specific βIII-tubulin. Another study using retinal whole mounts has demonstrated a >95% correlation between the number of TUJ1 $^+$ cells in the ganglion cell layer (GCL) of the retina and the number of RGCs visualized by retrograde labeling with FG after various experimental manipulations (Cui et al., 2003). Thus, although the rat's ganglion cell layer contains nearly equal numbers of RGCs and displaced amacrine cells (Perry and Walker, 1980), TUJ1 labeling reflects the number of surviving RGCs selectively. Three weeks after a PN graft, the number of TUJ1 $^+$ cells was twice as high after a low dose of Zymosan than in the saline-treated group (Fig. 4*a*) ($p < 0.001$). Cell survival was equally high regardless of whether Zymosan had been given the same day as nerve surgery or 3 d later. These data imply that cell survival and axon growth do not directly parallel

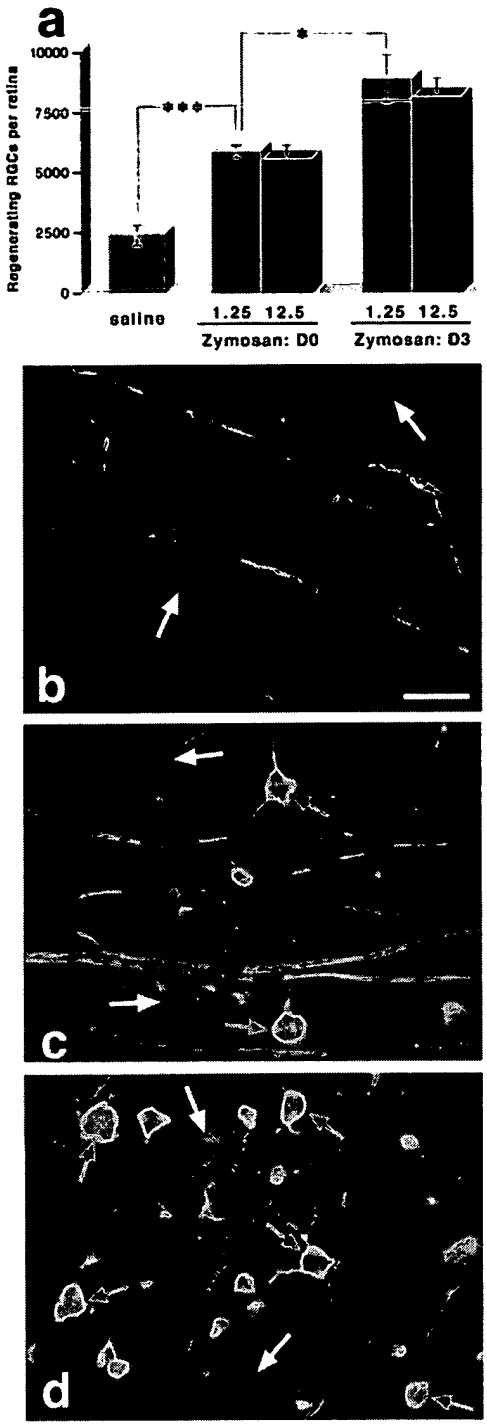

Figure 3. Axon regeneration in a peripheral nerve graft is enhanced by Zymosan. Fluorogold (*yellow* fluorescence) was injected into the distal end of a PN graft 3 weeks after a 1.5 cm segment of autologous peroneal nerve was sutured to the cut end of the optic nerve. *a*, Number of FG-labeled RGCs as a function of treatment. The concentration of Zymosan injected (micrograms per microliter) is indicated directly below the graph; the time of Zymosan injection relative to the day of grafting is shown below the bar (*D0*, same day as graft; *D3*, 3 d after optic nerve surgery). *b–d*, RGCs that had regenerated their axons the full length of the graft are shown with *red arrows*. In the retina, the TUJ1 antibody (*green* fluorescence) selectively labels RGC somata (*white arrows*) and processes (Cui et al., 2003). Animals receiving a PN graft and saline injections showed modest axon regeneration and modest TUJ1 staining (*a*, *b*). Zymosan injected at either of two doses on the same day as surgery increased the number of RGCs regenerating their axons through the graft relative to controls (*a*, *c*). *a*, *d*, Zymosan injections delayed by 3 d after grafting tripled the number of RGCs regenerating axons through the graft relative to controls. $*p < 0.05$; $***p < 0.001$. Scale bar: (in *b*) *b–d*, 50 μm.

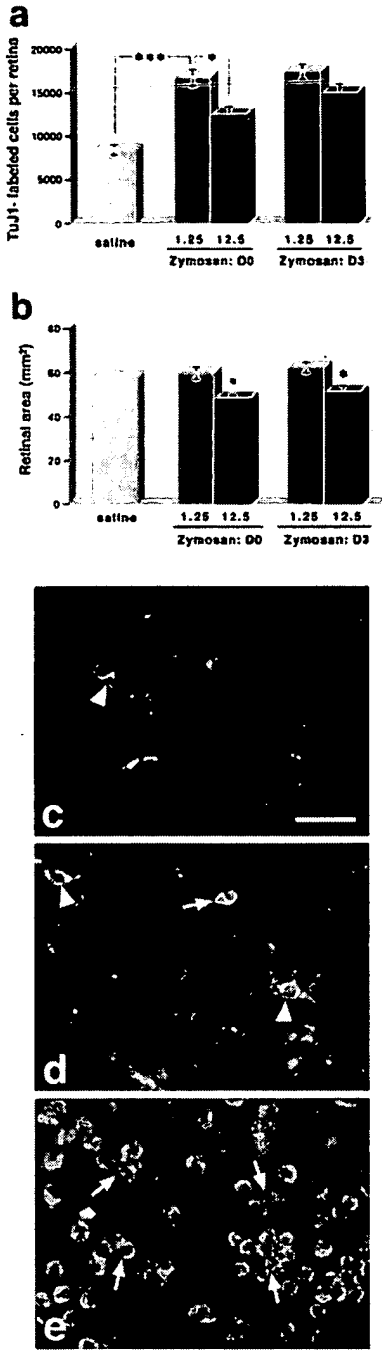

Figure 4. Effect of Zymosan dosage on RGC survival. *a*, Zymosan injections, either on the day of nerve crush (*D0*) or 3 d later (*D3*), increased the number of TUJ1$^+$ cells in the retina 3 weeks after a peripheral nerve graft. Zymosan resulted in more surviving TUJ1$^+$ cells when injected at a low concentration (1.25 μg/μl) than at a high concentration (12.5 μg/μl). *b*, The higher dosage of Zymosan diminished retinal size. *c*, Normal retina (flat mounted). No macrophages appear in the vitreous or around the GCL, although some ED-1$^+$ microglia are seen (*arrowhead*). *d*, Axotomy followed by a PN graft increases the number of ED-1$^+$ monocytes (*arrow*, *arrowheads*) in the retina only slightly. *e*, Zymosan injections (1.25 μg/μl) result in accumulation of ED-1$^+$ macrophages (*arrows*) in the vitreous and around the GCL. $*p < 0.05$; $***p < 0.001$. Scale bar: (in *c*) *c–e*, 50 μm.

each other, because (1) the group with Zymosan injected 3 d after nerve surgery showed considerably higher levels of axon regeneration than the immediate Zymosan-treated group, despite nearly identical survival levels, and (2) the higher dose of Zymo-

san resulted in lower cell survival than the lower dose, but similar levels of axon regeneration. The difference in RGC survival between the two doses was significant when both were given on the day of grafting ($p = 0.01$) but did not achieve statistical significance when both were given at day 3 ($p = 0.10$). The deleterious effect of the higher Zymosan dosage was also evident when measuring total retinal cross-sectional area (Fig. 4*b*) ($p < 0.01$). However, although higher doses of Zymosan decreased overall RGC survival, a greater proportion of surviving cells regenerated their axons through a PN graft.

The effects of Zymosan injections on monocyte activation after a peripheral nerve graft are shown in Figure 4 (*bottom*). As reported (Leon et al., 2000), few macrophages or microglia were detected in the GCL of normal retinas (Fig. 4*c*). After axotomy and a PN graft, a few activated monocytes appeared in the retina (Fig. 4*d*). Intravitreal Zymosan injection caused large numbers of macrophages to infiltrate the vitreous body and distribute over the GCL (Fig. 4*e*).

Bioactivity of macrophage-conditioned medium

To test further whether macrophages can mediate axon regeneration, we developed a primary cell culture model to investigate whether macrophages secrete factors that cause RGCs to grow axons. Dissociated cells of the adult rat retina were grown in a serum-free defined medium, and RGCs were identified by previous retrograde labeling with FG. In a typical experiment, RGC density after 3 d in culture was ~12 cells per square millimeter, representing a 60% survival rate from the time of plating; viability remained stable for up to 5 d (Y. Yin, unpublished observations).

As described elsewhere (Y. Li, N. Irwin, Y. Yin, L. I. Benowitz, unpublished observations), the mammalian vitreous humor contains a molecule similar to AF-1, a small factor (<1 kDa) that stimulates axon outgrowth in goldfish RGCs (Schwalb et al., 1995, 1996; Benowitz et al., 1998; Petrausch et al., 2000). When added to adult rat retinal cultures, vitreous-derived AF-1 had little effect by itself, but increased axon growth two- to threefold above control levels in the presence of forskolin (to elevate intracellular cAMP, $[cAMP]_i$) ($p < 0.05$) (Fig. 5*a*). RGC survival was unaffected by either AF-1 or forskolin (Fig. 5*b*). The axon-promoting effects of AF-1 remained at maximal levels after a 20-fold dilution, regardless of whether it was extracted from the vitreous of normal rats or from rats 1 week after lens puncture (data not shown). Thus, although AF-1 may help set the stage for axon regeneration *in vivo*, it cannot be considered a regulatory factor.

Macrophage-derived factors and AF-1 had an additive effect. As shown in Figure 5*a*, when tested alone, media conditioned by Zymosan-activated macrophages (containing proteins >3 kDa) enhanced axon regeneration two- to threefold above the baseline ($p < 0.001$). Media from macrophages that had not been treated with Zymosan in culture did not elevate growth above baseline, nor did Zymosan alone (data not shown). The effect of MCM was potentiated by AF-1 (growth in AF-1 + MCM vs MCM alone significant at $p < 0.01$) and was further augmented in the presence of AF-1 plus forskolin (Fig. 5*a*) ($p < 0.01$). MCM caused a slight decrease in cell survival that was not statistically significant ($p > 0.25$; ANOVA) (Fig. 5*b*).

Fractionation of MCM reveals that Zymosan-activated macrophages secrete both cytotoxic and axon-promoting factors. After separating components of MCM by ultrafiltration using a 30 kDa molecular weight cutoff membrane, the fraction containing proteins >30 kDa diminished axon outgrowth below baseline

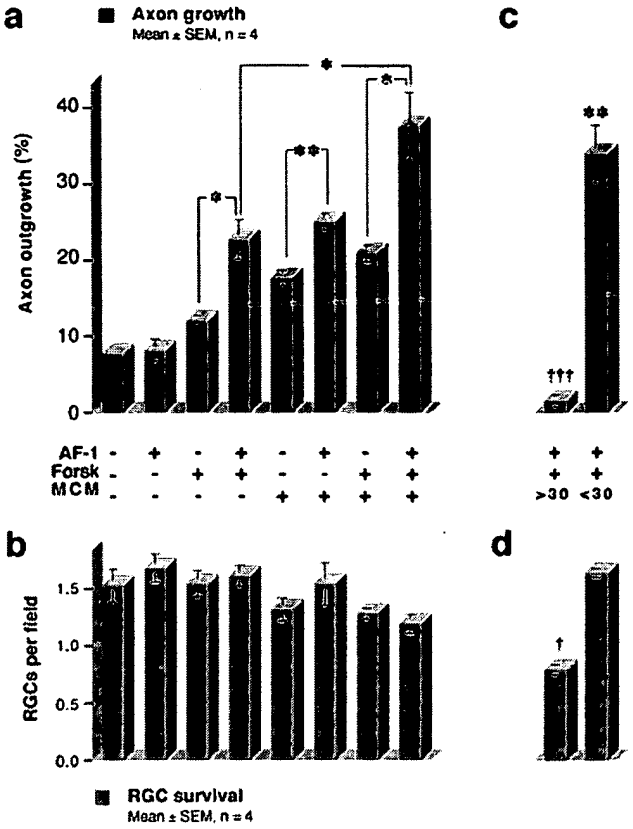

Figure 5. Macrophages secrete axon-promoting factors: additive effects with AF-1 and forskolin. *a*, AF-1, a low molecular weight constituent of the vitreous, stimulates axon growth in the presence of forskolin; forskolin itself has only modest effects. Conditioned media from Zymosan-activated macrophages (*MCM*) also stimulates axon growth. The addition of AF1 to MCM enhances growth above the level obtained with saturating concentrations of either one alone, and the further addition of forskolin results in even stronger outgrowth. *b*, None of these factors significantly alters RGC survival. *c*, *d*, When MCM was separated into fractions above and below 30 kDa, components <30 kDa stimulated axon growth, whereas components >30 kDa reduced axon outgrowth and cell survival relative to untreated controls. $^{*}p < 0.05$; $^{**}p < 0.01$; decrease significant at $^{\dagger}p < 0.05$; $^{\dagger\dagger\dagger}p < 0.001$.

levels (Fig. 5*c*) ($p < 0.001$) and decreased RGC survival (Fig. 5*d*) ($p < 0.05$). Higher concentrations of MCM >30 kDa caused all cells in culture to die (see Fig. 7). In contrast, the fraction of MCM containing molecules <30 kDa exhibited no toxicity and enhanced outgrowth above the baseline 1.8-fold compared with cells exposed to AF-1 + forskolin (Fig. 5*c*,*d*) ($p < 0.01$). Thus, the axon-promoting effects of macrophages appear to reside in molecules between 3 and 30 kDa, whereas factors with molecular weight ≥30 kDa are toxic.

As shown above (Fig. 1) and elsewhere, axon regeneration in mature RGCs correlates highly with enhanced expression of GAP-43 (Doster et al., 1991; Meyer et al., 1994; Schaden et al., 1994; Berry et al., 1996; Leon et al., 2000). After 3 d culture, cells were stained with a monoclonal antibody to GAP-43. Although 43% of FG-positive RGCs grown in defined medium alone showed some GAP-43 immunoreactivity, this was almost always weak (Fig. 6*a*,*b*). The addition of AF-1 plus forskolin stimulated 91% of RGCs to express intense GAP-43 immunoreactivity in their somata, axons, and growth cones (Fig. 6*c*,*d*). Axon growth was stimulated considerably further by the addition of MCM (Fig. 6*e*,*f*).

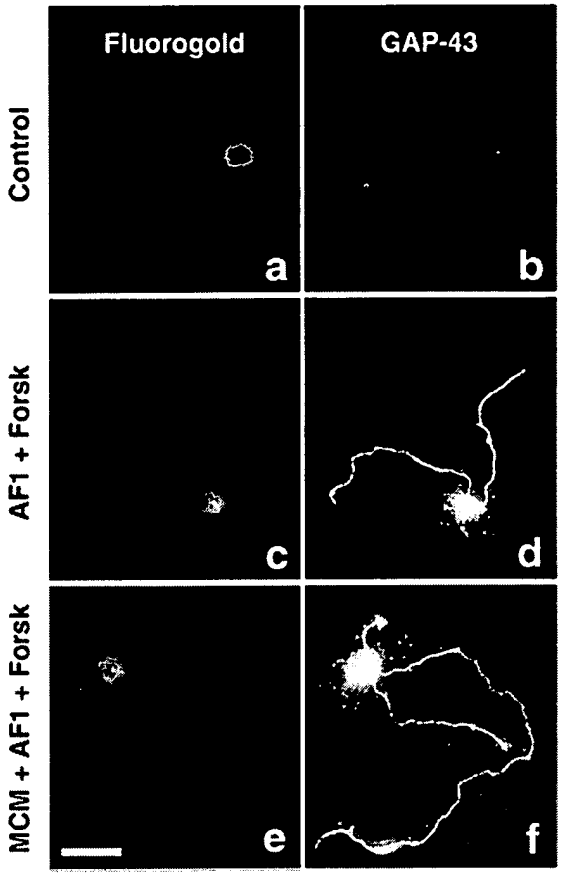

Figure 6. Axon outgrowth and GAP-43 expression in cultured RGCs. *a*, *c*, *e*, Fluorogold-labeled RGCs in culture; *b*, *d*, *f*, the same cells stained with an antibody to GAP-43. GAP-43 levels are low in RGCs cultured in defined media alone (*b*) but are high in cells exposed to AF-1 plus forskolin (*d*) or AF-1, forskolin, and MCM (*f*). The latter treatment induces the strongest outgrowth, with intense GAP-43 immunostaining in somata, axons, and growth cones. Scale bar, 50 μm.

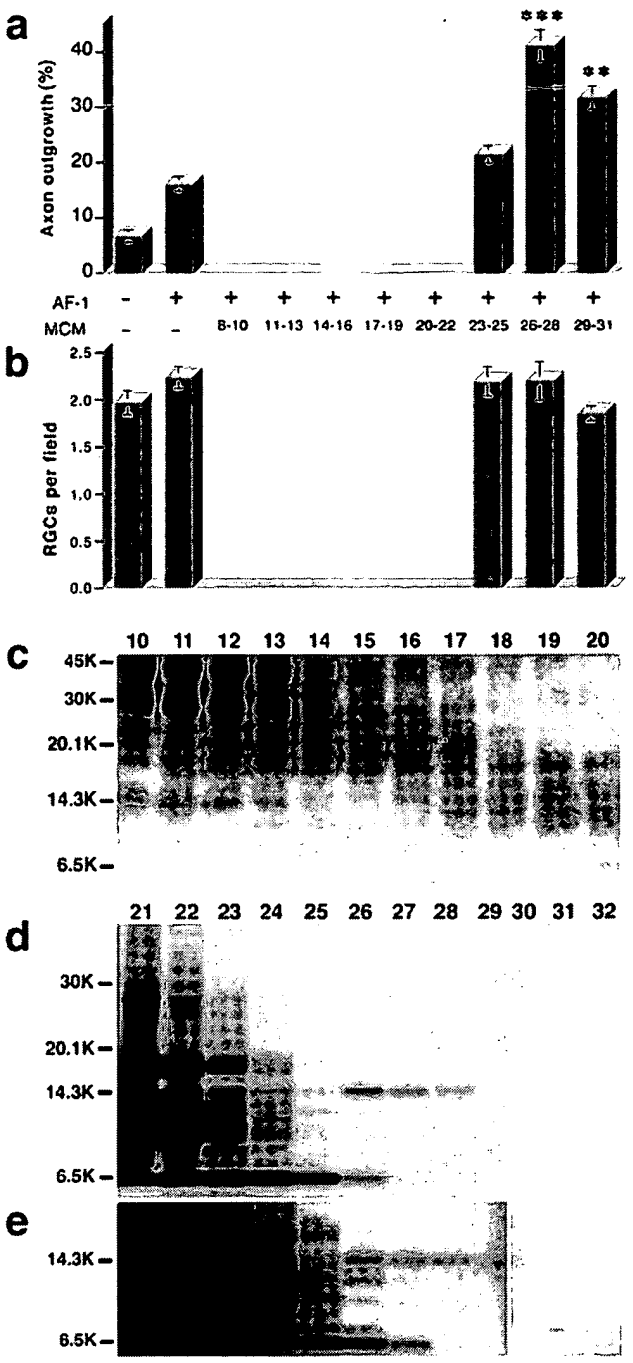

Figure 7. Bioactivity of MCM components. Media conditioned by Zymosan-activated macrophages (*MCM*) was separated by gel-filtration chromatography (Sephadex G-75). Pooled fractions were tested in dissociated retinal cultures in the presence of AF-1 (5%) plus forskolin (15 μM). *a*, Axon outgrowth. Fractions 26–31 more than doubled the percentage of RGCs extending axons relative to controls treated with AF-1 plus forskolin. *b*, Cell survival. Fractions before 23 were toxic to RGCs. *c*, Fractions 10–20, separated under reducing conditions by Tricine–SDS-PAGE and stained with Coomassie Brilliant Blue, show the presence of higher molecular weight components (only proteins below 45 kDa are shown), as well as smaller proteins that may have been parts of multimeric complexes. *d*, Fractions 21–32 separated on Tricine gels. *e*, Fractions 21–32 after silver staining. Fractions 26–29 include a prominent 14 kDa band. $^{**}p < 0.01$; $^{***}p < 0.001$.

Fractionation of macrophage-conditioned medium

To better define the axon-promoting and cytotoxic factors secreted by activated macrophages, we separated MCM by gel-filtration chromatography on a Sephadex G-75 column, which has a nominal separation range between 3 and 80 kDa. Polypeptides in the eluted fractions were separated electrophoretically on either 10% SDS-PAGE gels or 16% Tricine gels and visualized by Coomassie Brilliant Blue and then silver staining (Fig. 7*c–e*). The bioactivity of pooled subsets of fractions was tested in RGC cultures (Fig. 7*a*,*b*).

Fractions 10–22 were toxic to RGCs and other cells in culture (Fig. 7*b*). When analyzed by SDS-PAGE under reducing conditions, these fractions contained macromolecules with molecular weights above 20 kDa; proteins that migrated more rapidly were also seen, presumably resulting from the dissociation of multimeric complexes in the presence of SDS and β-mercaptoethanol (Fig. 7*c*). Fractions 23–25 contained proteins below 20 kDa and did not affect cell survival (Fig. 7*b*) or enhance growth above the level seen with AF-1 plus forskolin (Fig. 7*a*). Fractions 26–31 more than doubled the level of axon outgrowth seen with saturating concentrations of AF-1 plus forskolin (Fig. 7*a*) ($p < 0.001$). These fractions contained a 14 kDa band (Fig. 7*d*,*e*); bands between 6 and 8 kDa that were also present in these fractions were maximal in fractions 23 or 24, which did not promote growth (Fig. 7*a*). Ion-exchange chromatography was used to further separate proteins in fractions 26–31. Bioactivity continued to parallel the presence of the 14 kDa band (data not shown).

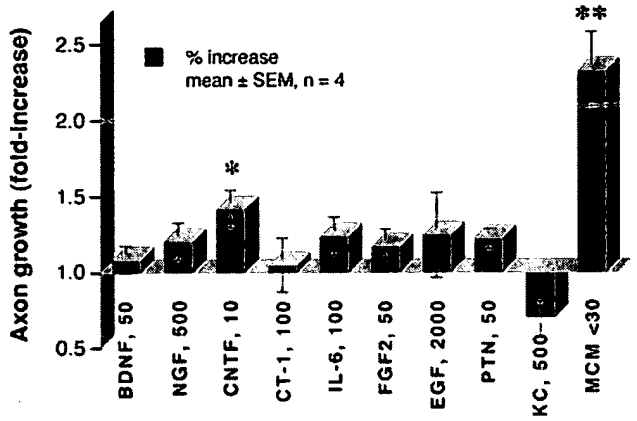

Figure 8. Axon-promoting effects of defined trophic factors. Trophic factors were tested in retinal cultures at concentrations 5–10 times higher than necessary to achieve saturated effects (concentrations used are shown in nanograms per milliliter). Data from different experiments were normalized to show axon growth relative to that obtained with 5% AF-1 plus 15 μM forskolin (=100%). CNTF exerted significant axon-promoting effects on RGCs; these effects were lower than those achieved with the low molecular weight fraction of Zymosan-activated macrophage conditioned media (*MCM <30*). See Material and Methods for abbreviations of trophic factors used. $*p < 0.05$; $**p < 0.01$.

Defined trophic factors

We investigated whether identified trophic factors with molecular weights in the 14 kDa range would mimic the axon-promoting effects of the macrophage-derived molecule(s). The factors that we tested included the neurotrophins BDNF and NGF, the cytokines CNTF, CT-1, and IL-6, along with GDNF, EGF, FGF-2, pleiotrophin, and the chemokine KC/GRO/CXCL1. With the exception of CNTF, none of these factors stimulated outgrowth (Fig. 8). CNTF promoted axon growth in cultured RGCs starting at a concentration of 1 ng/ml, and the effect saturated at 10 ng/ml (data not shown). CNTF at a saturating concentration increased outgrowth 1.4-fold above the level induced by AF-1 + forskolin ($p < 0.05$). The low molecular weight fraction of MCM had a considerably stronger effect (Fig. 8).

Although none of the defined growth factors tested enhanced RGC survival, NGF caused a modest but significant decrease (data not shown). NGF had similar deleterious effects on immunopurified postnatal day 8 RGCs (Jo et al., 1999). This effect may be caused by the activation of p75LNTFR in the absence of trkA signaling (Frade et al., 1996; Frade and Barde, 1998).

Discussion

We show here that macrophages can stimulate mature RGCs to regenerate their axons well beyond an injury site into the distal optic nerve, an environment that is normally hostile to axon growth. Dramatic optic nerve regeneration has previously been achieved by injuring the lens (Leon et al., 2000; Fischer et al., 2001) or implanting a peripheral nerve fragment into the vitreous (Berry et al., 1996). Injuring the lens leads to macrophage infiltration into the eye (Leon et al., 2000), and activating macrophages with Zymosan produces even stronger axon regeneration than lens injury [comparing these results with those of Leon et al. (2000)]. The beneficial effects of a peripheral nerve implant may likewise involve macrophages, which were abundant in those implants (Berry et al., 1996). In a more permissive environment, intravitreal macrophage activation stimulated RGCs to regenerate axons rapidly through a peripheral nerve graft (this study), thus mimicking the effects of lens puncture (Fischer et al., 2000). The possibility that macrophages play an important role in these various instances of optic nerve regeneration is further supported by the results of our cell culture studies.

Macrophages secrete numerous factors, some with beneficial effects on neurons (e.g., BDNF, IL-6, PDGF, GDNF) and some with deleterious effects (e.g., TNF-α and IL-1β) (Ballou and Lozanski, 1992; Dougherty et al., 2000; Leskovar et al., 2000; Rappolee and Werb, 1992). When separated by size, macrophage-derived factors >30 kDa were found here to be cytotoxic to retinal cells in culture, whereas molecules of 10–20 kDa caused RGCs to regenerate their axons. Axon growth was correlated with the presence of a protein secreted by macrophages with an apparent size of 14 kDa. In culture, several neurotrophins (BDNF, NGF), cytokines (cardiotrophin-1, IL-6), FGF-2, EGF, chemokine KC, and pleiotrophin all failed to stimulate growth. CNTF induced significant outgrowth, as expected (Cui et al., 1999; Jo et al., 1999; Cui and Harvey, 2000), but its effects were weaker than those of the macrophage-derived factor. CNTF, BDNF, and GDNF injected intravitreally were unable to stimulate axon regeneration in the optic nerve (Leon et al., 2000; Yin, unpublished observations).

At the site of a peripheral nerve injury, macrophages promote tissue healing through phagocytosis of cellular debris and by providing signaling molecules that activate other cells. In the CNS, the macrophage response is normally attenuated. However, when peripherally activated macrophages are transferred to the CNS, they can enhance CNS regeneration, presumably by phagocytosing inhibitory myelin components (David et al., 1990; Lazarov-Spiegler et al., 1996; Rapalino et al., 1998). Macrophages are normally abundant at the site of an optic nerve crush (Berry et al., 1996), but these are unlikely to provide adequate trophic support to RGCs in view of the regenerative failure that occurs normally. Our results show that macrophage activation closer to RGC somata produces far more dramatic effects.

The production of both positive- and negative-acting factors by macrophages may help explain the relationship between axon regeneration and the timing of macrophage activation. The number of ED-1$^+$ macrophages in the eye rises continuously over the first week after lens injury (Leon et al., 2000). We therefore predicted that if macrophages were induced early, appropriate factors would be present at the time of axotomy to enable RGCs to regenerate their axons across the injury site before a scar forms. Unexpectedly, macrophage activation 7 d before nerve injury produced no growth, whereas activation 3 d after nerve injury produced the strongest growth into the distal optic nerve or into a peripheral nerve graft. These findings imply that (1) the responsiveness of RGCs to factors induced by macrophage activation increases several days after axotomy, possibly because of delayed expression of a trophic factor receptor or of its downstream signaling elements, and (2) the net effect of macrophage activation is most favorable shortly after induction and then becomes unfavorable. This could be a concentration-dependent effect or could reflect differential expression patterns for positive- versus negative-acting factors. If macrophage activation occurs too late, RGCs may have already initiated an irreversible apoptotic program, whereas early activation may leave too high a concentration of negative factors by the time RGCs become responsive.

In culture, the survival of early postnatal RGCs isolated by immunopanning requires several growth factors plus elevated $[cAMP]_i$ (Meyer-Franke et al., 1995). The survival of mature RGCs in our cultures, even without these factors, may be attributable to trophic factors provided by other cell types or to particular components of our defined media. Mature RGCs can survive and extend neurites within explants (Meyer et al., 1994; Fischer et

al., 2000) on a preformed layer of neonatal cortical astrocytes (Wigley and Berry, 1988) or with fetal bovine serum and certain antibodies as substrates in the culture medium (Gaudin et al., 1996; Luo et al., 2001). The dissociated cultures used here have the advantage of enabling us to quantify the survival, morphology, and neuritogenesis of individual RGCs under well controlled conditions.

Our earlier studies in goldfish showed that non-neuronal cells of the optic nerve secrete a low molecular weight factor with potent axon-promoting effects on RGCs (Schwalb et al., 1995, 1996). A molecule with similar biophysical properties and bioactivity is abundant in the mammalian vitreous, regardless of whether the lens has been injured (Li, Irwin, Yin, and Benowitz, unpublished observations). As shown here, this factor, which we have termed AF-1, exerts axogenic effects on mature rat RGCs in culture in the presence of forskolin. *In vivo*, AF-1 alone is clearly not sufficient to stimulate RGCs to regenerate their axons after injury, in view of the normal failure of mature rat RGCs to do so. However, as shown in our culture studies, AF-1 strongly potentiates the effect of the macrophage-derived factor(s) and hence may play a similar permissive role *in vivo*.

A multiplicity of factors normally limits CNS regeneration. Three oligodendrocyte proteins, Nogo-A, MAG (myelin-associated glycoprotein), and OMgp (oligodendrocyte/myelin glycoprotein), restrict the growth of axons over myelin via their interaction with the Nogo-66 receptor (Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002). Although macrophage activation stimulated stronger axon growth in a PNS graft than in the optic nerve, this difference may not be caused solely by differences in glial environment. After an optic nerve crush, damaged nerve endings confront myelin debris, along with a glial scar and a cavity. The importance of the glial scar in limiting axon regeneration is illustrated by several recent studies (Davies et al., 1997; Stichel et al., 1999; Bradbury et al., 2002) [but see Weidner et al. (1999)]. If the adult rat optic nerve is cut rather than crushed and the two ends are then sutured together, a significant number of RGCs regenerate their axons to the superior colliculus after lens injury (Fischer et al., 2001). Thus, the inhibitory effects of myelin are clearly not insuperable. Perhaps the activation of a growth program in RGCs by macrophage-derived factors desensitizes RGC growth cones to myelin.

One protein that is strongly induced by macrophages is GAP-43. GAP-43 induction correlates with successful axon regeneration in the optic nerve and elsewhere (Skene, 1989; Doster et al., 1991; Benowitz and Routtenberg, 1997), and forced overexpression of this protein and the related submembrane cytoskeletal protein, CAP-23, enhances axon regeneration in the CNS and PNS (Aigner et al., 1995; Bomze et al., 2001). Hence, GAP-43 may be one contributor to the regeneration seen here. Another factor that can influence the growth state of the neuron is $[cAMP]_i$. Elevated $[cAMP]_i$ enables neurons to respond to growth factors (Meyer-Franke et al., 1995, 1998) and overcome multiple inhibitory cues (Ming et al., 1997; Song et al., 1998; Cai et al., 1999). In our studies, elevated $[cAMP]_i$ potentiated the effects of AF-1, although not of the macrophage-derived factor per se. Finally, it has been suggested that RGCs irreversibly switch from an axon-growth program to a dendritogenic program shortly after birth as a result of an amacrine cell-derived factor (Goldberg et al., 2002). However, the remarkable regeneration of RGC axons achieved here and in other studies (Aguayo et al., 1991; Berry et al., 1996; Cui and Harvey, 2000; Fischer et al., 2000, 2001; Leon et al., 2000) argues that this switch is not irreversible.

Whatever downstream mechanisms are involved, it is clear that one or more factors released by macrophages enable adult mammalian RGCs to overcome some of the barriers that normally restrict axon regeneration in the CNS. Identification of the active factor(s) may permit us to selectively mimic the pro-regenerative effects of macrophages without the cytotoxic effects, and hence possibly enhance outcome after injury in the visual system and elsewhere in the CNS.

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